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RD 114 Virus-Specific Sequences in Feline Cellular RNA: Detection and Characterization

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RNA extracted from cat cells contains sequences homologous to RD-114 viral RNA. The sequences are measured by molecular hybridization with a single-stranded DNA probe synthesized by the virion polymerase using the endogenous viral RNA as template. Viral-specific RNA has been detected in all cells of cat origin tested thus far, but not in cells of other animals, except for the virus-producing human rhabdomyosarcoma cell, RD-114. The extent of hybridization of the DNA probe to cellular RNA was equivalent to that obtained with viral 70*S* RNA indicating that an equal extent of viral specific sequences is present in all cat cells as well as in RD-114 cells. The amounts of this viral RNA reach approximately 100 copies per cell in cat cells, while virus-producing RD-114 cells contain about 1,000 copies per cell. The viral RNA is present in cat cells in two distinct sizes of about 35*S* and 18*S*, whereas in RD-114 cells virus-specific RNA is quite heterogeneous in size.

Since the discovery of the RD-114 virus (21) which was isolated from a human rhabdomyosarcoma cell line after transplantation in a fetal cat, it has attracted profound interest based on its qualification as a putative human cancer virus. Biological and biochemical studies of the virus have shown it to be distinct from all other known type C RNA viruses. These include observations of immunological uniqueness of virion group-specific (gs) antigens (24), and of RNA-dependent DNA polymerase (20, 30), lack of reciprocal interference with feline viruses (13, 21), and characteristic syncytia formation on an RSV-transformed human cell line (26). These procedures do not, however, specify species of origin.

The discovery of RNA-dependent DNA polymerase in RNA tumor viruses (2, 34), which makes it possible to synthesize highly radioactive DNA complementary to viral RNA, has provided one approach to this problem. Because of the highly specific complementarity of the DNA product with viral RNA (32), this DNA has been used successfully as a probe to detect virus-specific RNA sequences in virus-infected or transformed cells by molecular hybridization (4, 9, 11, 15, 18, 36). Recently, Hayward and Hanafusa (16) reported the presence of low levels of viral RNA in uninfected normal avian embryos while Parks et al. (25) reported similar results using normal mouse cells.

To assess the natural host of RD-114 virus, similar approaches have been taken to detect virus-specific RNA sequences in various cells of different origin (23). Extensive base sequence homology was detected between the RD-114 DNA probe and cellular RNA of cat cell origin. Sequences homologous to RD-114 viral RNA have also been detected specifically in cellular DNA from normal cat organs by using RNA-DNA hybridization (3, 22) and DNA-DNA hybridization (27).

Recent attempts to isolate endogenous type C viruses from cat cells resulted in induction, either by IUdR or spontaneously, of a type C virus from an established cat cell line, which shared identical biological and biochemical characteristics with RD-114 virus (19, 28). This, along with the hybridization data, indicates that the RD-114 virus is a feline endogenous virus, belonging to an entirely different group of feline type C viruses from the known ones, and which can be induced or amplified by contact with susceptible human or primate cell lines.

Further characterization of the RD-114 specific RNA in cat cells is reported here.

MATERIALS AND METHODS

Materials. Pancreatic RNase A, RNase-free DNase I, and calf thymus DNA were obtained from Worthington Biochemicals Co. (Freehold, N.J.). Pronase (B grade), Actinomycin D, and dithiothreitol (Cleland's reagent, DTT) were obtained from Calbiochem (Los Angeles, Calif.). α-Amylase, type IV-A from *Aspergillus oryzae* was obtained from Sigma Chemical Co. (Saint Louis, Mo.). Poly A, Poly G, and *Neurospora crassa* conidia were obtained from Miles Laboratories (Kankakee, Ill.). Hydroxyapatite (Bio Gel HT) and sodium dodecyl sulfate were obtained from Bio-Rad (Rockville Center, N.Y.). Dimethyl sulfoxide, spectrophotometric grade (DMSO), and sucrose, special density gradient grade, were obtained from Schwarz-Mann (Rockville, Md.). Cs_2SO_4, optical grade, was obtained from the Harshaw Chemical Co. (Solon, Ohio). Nonidet P-40 (NP-40) was obtained from the Schell Chemical Co. (Great Britain). Dimethyl-D^6-sulfoxide was obtained from ICN (Irvine, Calif.). ^3H-TTP, ^3H-dCTP, and carrier-free $H_3P^{32}O_4$ were obtained from New England Nuclear Corp. (Boston, Mass.). All the other chemicals used were reagent grade commercial products.

Cells. Tissue cultures were grown in Eagle minimal essential medium (MEM) with Earle salts (Flow Laboratories, Inc.), supplemented with 10% calf serum and 100 U of streptomycin and penicillin per ml at 37 C in 32-oz (about 960 ml) culture bottles. The cells were allowed to grow to confluency with medium replenishments at 4- to 7-day intervals. The description of the cells used is given in Table 2.

All fresh organs from normal animals were obtained from Bionetics, Inc. (Bethesda, Md.), and either immediately used for RNA extraction or quickly frozen in dry ice and stored at −80 C until use. Cat hepatomas were kindly supplied by Howard Charman (U.S.C., Department of Pathology).

Extraction of cellular RNA. Cells in monolayer cultures were washed twice with phosphate-buffered saline (PBS) (Flow Laboratories, Inc.), scraped off into isotonic buffer (0.25 M sucrose, 0.01 M Tris-hydrochloride, pH 7.4, 0.01 M KCl, 0.01 M $MgCl_2$), and collected by a centrifugation at 3,000 rpm for 10 min. RNA was immediately extracted by the SDS-hot phenol method (29). The ethanol precipitates were collected by a centrifugation at 13,000 rpm for 30 min, and dissolved in 0.01 M Na-acetate, pH 5.2, 0.05 M NaCl. The solution was made to 0.005 M with $MgCl_2$, and treated with 10 μg of DNase I per ml at 37 C for 15 min. EDTA and SDS were then added to 0.01 M and 0.5%, respectively, and the solution was treated with 50 μg of Pronase per ml (self-digested at 37 C for 2 h) at 37 C for 60 min. It was then deproteinized with phenol three times at room temperature, followed by ethanol precipitation. The precipitates were finally dissolved in 0.1 × SSC. The concentration of RNA (mg/ml) was estimated by dividing the absorbance at 260 nm by 24.

Cells in suspension cultures were harvested by a centrifugation, washed with PBS, and the RNA was extracted as described above.

Tissues of fresh organs were minced with scissors, suspended in PBS, and homogenized with Polytron (PT-10ST, Brinkman Instruments, Inc.) at setting 3 for 1 min in ice. The suspension was diluted 10-fold with 0.01 M Na-acetate buffer, pH 5.2, and RNA was extracted as described above.

Viruses. RD-114 virus was grown in monolayer cultures of RD-114 cells (21). Feline leukemia virus (FeLV) (Theilen strain) was obtained from a chronically infected cat lymphocytic cell suspension culture (35), and Rauscher leukemia virus (RLV) from a JLS-V9 monolayer culture (38).

The culture supernatant fluids were filtered through a membrane filter (type RA, 1.2 μm pore size, Millipore Corp.) and chilled in ice. Viruses were harvested from the filtrates by a continuous flow isopycnic centrifugation either with the model K, Mark II centrifuge (Electro-Nucleonics), or with the CF-32 rotor in a Spinco L-350 centrifuge. The virus band was localized by absorbance at 260 nm and complement-fixation tests for the virus gs antigen (24). The pooled virus fractions were diluted with 0.01 M Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 0.001 M EDTA (TSE) to less than 20% sucrose, and rebanded in a Ti-15 zonal rotor (Beckman Instruments) through 25% sucrose layer onto 50% sucrose cushion in TSE for 16 h at 4 C. The virus band was collected and used either directly or after repelleting by a centrifugation to remove sucrose.

RD-114 virus obtained from Pfizer, Inc. (Maywood, N.J.) and feline sarcoma virus (FeSV) (Gardner strain) and RLV obtained from Electro-Nucleonics (Bethesda, Md.) were also used.

Viral RNA. The high molecular weight 70*S* RNA was prepared from the purified virus (1 to 5 mg of protein per ml) by lysing with 1% SDS, and sedimenting through a linear sucrose gradient of 15 to 30% (wt/vol) in TSE containing 0.01% SDS at 40,000 rpm for 3 h in a Beckman SW 41 rotor. The 70*S* region was pooled, deproteinized with 80% phenol (pH 5.2) three times at room temperature, and precipitated repeatedly with 2 volumes of ethanol in 0.2 M NaCl. The concentration was estimated by the absorbance at 260 nm as described above. A typical sedimentation profile is shown in Fig. 1A.

Viral DNA probe. In order to obtain quantitative hybridization, it was found necessary to use single-stranded DNA probes purified from the viral 70*S* RNA hybrids in the endogenous reaction mixture. The endogenous polymerase reaction mixture consisted of 100 mM glycine buffer, pH 8.3, 5 mM $MgCl_2$, 50 mM NaCl, 10 mM DTT, 50 μM each of deoxyribonucleoside triphosphates, one ^3H-labeled deoxyribonucleoside triphosphate at 10 μM (usually 45 to 50 Ci/mmol ^3H-TTP), 0.01% NP-40, and purified virus (0.2 to 1 mg of protein per ml). The mixture was incubated at 37 C for 60 min and the reaction was stopped by the addition of 1% SDS. The mixture was directly layered on a 10-ml column of 15 to 30% (wt/vol) linear sucrose gradient in TSE containing 0.01% SDS, and centrifuged at 40,000 rpm for 3 h in an SW 41 rotor. Fractions of about 0.6 ml were collected from the bottom, and samples were removed to measure acid-precipitable counts. A typical sedimentation profile of ^3H-DNA is shown in Fig. 1B. The 70*S* regions were pooled, made to 0.5% SDS and 0.01 M EDTA, and then extracted with an equal volume of 80% phenol, pH 7.8, for 5 min at room temperature, followed by chloroform: iso-amyl alcohol (24:1) for an additional 5 min. The organic phase was discarded after a centrifugation, and the extraction was repeated two more times with chloroform. The aqueous phase was finally removed, treated with 0.2 N NaOH at 80 C for 20 min to destroy RNA, chilled in ice, and neutralized with 1 N HCl. The DNA was precipitated

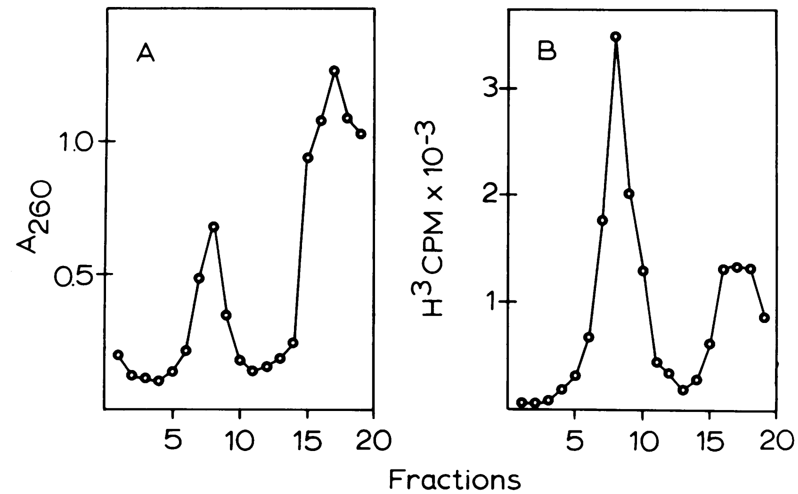

FIG. 1. *Sedimentation profiles of RD-114 viral RNA (A) and the DNA product of the endogenous polymerase reaction (B). A 2-ml amount of concentrated virus suspension was mixed with 0.1 ml of 20% SDS (A), immediately layered on 10 ml of a 15 to 30% (wt/vol) linear sucrose gradient, and centrifuged at 40,000 rpm for 3 h at 4 C in a SW-41 rotor. Sedimentation was from right to left. (B), Endogenous polymerase reaction mixture (2 ml) with ³H-TTP (51 Ci/mmol) was incubated at 37 C for 60 min, mixed with 0.1 ml of 20% SDS, and centrifuged as in Fig. 1 A. Samples (25 μliters) of each fraction were precipitated with cold 6% trichloroacetic acid in the presence of 50 μg of calf thymus DNA and counted. The position of 70S RNA in both figures is in fraction 8.*

with 2 volumes of ethanol in the presence of 100 μg of yeast RNA repeatedly, and finally dissolved in 0.1 × SSC. The concentration of DNA was estimated from the acid-precipitable counts of the preparation, on the basis of the specific activity of ^{3}H-labeled substrate.

Also, a total single-stranded DNA product (fractionated on a hydroxyapatite column) made during an endogenous polymerase reaction in the presence of 100 μg of actinomycin D per ml was prepared (12). Actinomycin D selectively inhibits the DNA-dependent DNA polymerase activity, yielding the product of a complete and uniform transcript of viral RNA (12).

Single-strand specific nucleases. The S-1 enzyme from *A. oryzae* was prepared from α-amylase, according to the method of Sutton (33). The specific activity was 69,300 U/A_{260} unit. The enzyme preparation was stored frozen at −20 C, and diluted 10-fold before use with 25% glycerol in 0.01 M Na-phosphate, pH 6.8.

Hybridization. Hybridization was carried out in 100 μliters of 0.3 M NaCl, 0.02 M Tris-hydrochloride, pH 7.2, 0.001 M EDTA, and 0.1% SDS. The mixture of a given amount of RNA and 500 to 1,000 counts/min of ^{3}H-DNA probe in 0.1 × SSC was boiled for 3 min, chilled in ice, and brought to 0.3 M NaCl; the mixtures were then incubated at 67 C for a given time.

Assay by S-1 enzyme. Hybridized mixtures (usually 100 μliters) were diluted to 2.5 ml to give final concentrations of 0.034 M Na-acetate, pH 4.5, 0.14 mM $ZnCl_2$, 0.18 M NaCl, and 10 μg of denatured calf thymus DNA per ml. The enzyme of 25 μliters was added (26 × 10^3 U/ml) and the mixtures were incubated at 45 C for 120 min. DNA resistant to nuclease digestion was precipitated with cold 10% trichloroacetic acid in the presence of 50 μg of calf thymus DNA, collected onto type HA Millipore filters, washed with 6% trichloroacetic acid, and counted in Liquifluor-toluene by using a Beckman LS-250 liquid scintillation system.

Glycerol gradient analysis. Linear gradients of 15 to 30% (wt/vol) glycerol which was filtered twice through a Nalgene filter, were made in TSE containing 0.05% SDS. About five to ten A_{260} units of RNA were loaded on the top of the gradients and centrifuged at 25,000 rpm for 14 h at 5 C in an SW 41 rotor. Fractions (0.5 ml) were collected from the bottom and 75-μliter samples were directly hybridized to DNA probes.

DMSO gradient analysis. Linear gradients of 99% (normal and deutrated) dimethylsulfoxide in 4.6 ml were prepared (1), and loaded with about three A_{260} units of RNA. Sedimentation was at 40,000 rpm for 13 h at 25 C in an SW 65 rotor, using uniformly labeled RD-114 cellular ^{3}H-RNA as a marker. Fractions of 0.25 ml were collected from the bottom and RNA was precipitated with 2 volumes of ethanol in the presence of 0.2 M NaCl and 50 μg of calf thymus DNA. The precipitates were collected by centrifugation, rinsed with 70% ethanol, and redissolved in 0.1 × SSC.

RESULTS

Characteristics of the DNA probe. The DNA probe used in this study was prepared as a single-stranded form from a complex with viral 70S RNA, and was completely hydrolyzed by the S-1 single-strand specific nuclease. Complete hydrolysis of the probe was also obtained when the hybridization mixture with added

RNA was immediately cooled, stored frozen, and assayed later with S-1. There was a slight increase of S-1 resistance observed when the DNA probe was incubated at 67 C without RNA or with bacteriophage MS2 RNA, probably due to a time-dependent self-annealing reaction. On the other hand, more than 80% of the radioactivity in the DNA probe became S-1 nuclease resistant after hybridization with the homologous RD-114 viral 70S RNA.

In this study, the levels of hybridization were principally assayed with a single-strand specific nuclease S-1 prepared from *A. oryzae*. Similar results were obtained with other methods, namely hydroxyapatite fractionation, Cs_2SO_4 density equilibrium centrifugation, or another single-strand specific nuclease purified from *N. crassa*. As shown previously (23), the hybrids thus formed between the DNA probe and cellular RNA from RD-114 or Crandell cells showed very similar thermal stability compared with that obtained with RD-114 viral RNA, indicating a high degree of the sequence complementarity between the probe and cellular RNA with minimal mismatches.

Specificity of hybridization. Table 1 shows the hybridizations of various cellular and viral RNAs with three different viral DNA probes, RD-114, FeLV, and RLV. The species specificity of the probes is evident. Cellular RNA from virus-shedding cells hybridized extensively with their respective viral DNA probe, whereas the other cellular RNA preparations hybridized to a minimal extent, except for RNA from cat cells, which hybridized with the RD-114 probe. The hamster cell line, HT-1, a nonproducer MSV transformant, is known to contain a low level of RNA hybridizable to ^{3}H-DNA product of MSV (15), as confirmed here (Table 1). There was a low degree of cross-hybridizability observed with some viral 70S RNA, notably between RD-114 and FeSV, and between FeLV and RLV. The hybridization of the RD-114 DNA probe with FeSV RNA probably reflects the incorporation of RD-114-specific RNA into the virus particles grown in cat cells, since such hybridization was not observed with RNA extracted from FeLV which was grown in RD cells (Okabe et al., manuscript in preparation). There were low levels of cross-hybridization between FeLV and RLV, and between the Gibbon Ape virus and RLV (Okabe et al., manuscript in preparation). The low but significant amounts of hybridization observed with RNA from virus shedding cells, FL-74 (FeLV), and C3H (MSV), with heterologous DNA probes are presumably based on a low level of similar (not necessarily identical) nucleic acid sequences in the various viruses.

Widespread presence of RD-114 specific RNA in cat cells. When the RD-114 virus DNA probe was hybridized to cellular RNA prepared from a cat cell line, CRFK, very high levels of hybridization were observed (23). This cell had no detectable RD-114 gs antigen when assayed by complement-fixation tests, but a type C virus identical with RD-114 virus was induced by IUdR treatment followed by mixed culture with RD and other human and primate cells (19, 28). The presence of RD-114-specific RNA

TABLE 1. *Cross-hybridization using several DNA probes*[a]

RNA		^{3}H-DNA (input)					
Source	Amount (μg/0.1 ml)	RD-114(1,343 counts/min)		FeLV(1,197 counts/min)		RLV(897 counts/min)	
		Counts/min	%	Counts/min	%	Counts/min	%
Cells							
RD-114	71	990[b]	106	32	3	0	0
RD	154	0	0	17	2	0	0
FL-74	97	708	76	1078	111	68	10
Cat kidney	129	176	19	28	3	0	0
Crandell	32	717	77	31	3	0	0
C3H(MSV)	56	0	0	195	20	591	85
C3H	46	0	0	13	1	87	13
HT-1	100	0	0	50	5	231	33
Virus							
RD-114, 70S	0.3	931	100	21	2	23	3
FSV, 70S	0.2	88	10	968	100	94	14
RLV, 70S	0.2	25	3	107	11	695	100
MS-2	10.0	(116)		(44)		(46)	

[a] Hybridization was at 67 C for 16 h.

[b] All values after subtraction of the background obtained with MS-2 RNA.

was not confined to the CRFK cell; cat hepatoma samples and even normal cat livers contained significant amounts of RNA hybridizable to the RD-114 probe. The extent of hybridization of the DNA probe approximated that obtained with RD-114 viral 70*S* RNA or with RNA from RD-114 cells. The DNA probe did not contain transcripts of the host-cell RNA since RNA from the virus-free parental cell line, RD, did not hybridize above background levels. When the hybridization study was extended to other cellular RNAs from cat and other mammals, it was found that all cells of cat origin examined contained significant amounts of RNA hybridizable to the RD-114 DNA probe (Table 2). These include two independent sources of the Crandell cell line obtained from P. Sarma (CRFK) and from J. Gillespie (Cornell University) (CC), a lymphocytic cell line FL-74 (35), normal cat embryo fibroblasts, normal liver, spleen, kidney, and two different cat hepatoma preparations obtained from H. Charman (U.S.C.). The hybridization was highly specific for RNA of cat cells, and was not observed with RNA from cells of human, monkey, cow, rat, mouse, and hamster origin. The Raji cell, a human lymphoblastoid cell line, was negative for this RNA, but became highly positive upon infection with RD-114 virus (data not shown).

The concentration of this RNA varied from cell to cell; freshly removed organs containing much less RD-114-specific RNA as compared to cells in tissue culture. At an RNA concentration of 2 to 3 mg/ml in the hybridization mixture, RNA from liver, spleen, and kidney converted only 35 to 45% of the DNA probe into hybrid form after 16 h of incubation, and the hybridiza-

TABLE 2. *Hybridization of various cellular RNAs with the DNA product of RD-114 virus*

Cell	Origin	Type of cell	Virus	Amount of RNA (μg/0.1 ml)	Hybridization[a]	
					Counts/min	%
RD-114	Human	Rhabdomyosarcoma	RD-114	33	498	91
RD	Human	Rhabdomyosarcoma		229	0	0
HeLa	Human	Carcinoma		238	0	0
Raji	Human	Lymphoblastoid	(EBV)	286	0	0
SV-80	Human		Gibbon Type C	201	0	0
AGM	Monkey	Normal liver		197	0	0
Rhesus	Monkey	Normal liver		147	0	0
Calf	Cattle	Normal spleen		605	0	0
Crandell	Feline	Normal kidney cell		140	476	87
(CC)	Feline	Normal kidney cell		104	340	73
Cat embryo	Feline	Normal fibroblast		74	285	62
FL-74	Feline	Leukemic cell	FeLV	103	519	95
Cat	Feline	Normal liver		275	251	46
Cat	Feline	Normal spleen		209	226	41
Cat	Feline	Normal kidney		193	200	36
Cat tumor	Feline	Hepatoma (69024)		124	447	81
Cat tumor	Feline	Hepatoma (69203)		143	463	84
XC	Rat	RSV-transformed	(RSV)-NP	118	2	0.4
Rat	Rat	Normal kidney		441	0	0
C3H	Mouse	Normal fibroblast		68	1	0.2
C3H(MSV)	Mouse	MSV-transformed	MSV/RLV	84	0	0
Mouse	Mouse	Normal kidney		276	0	0
HT-1	Hamster	MSV-transformed	(MSV)-NP	150	1	0.2
Rat t-RNA				75	3	0.5
Poly A				20	0	0
Poly G				20	0	0
RD-114, 70*S* viral RNA				0.1	549	100

[a] Each RNA preparation was hybridized with ^{3}H-DNA product of RD-114 (660 counts/min). Hybridized counts were obtained as trichloroacetic acid-precipitable counts after S1 nuclease digestion, and given as a net counts per minute after subtracting the background (59 counts/min). The percentage of hybridization was normalized, taking the counts per minute hybridized with RD-114 70*S* RNA as 100%.

tion levels of the RNA from tissue cultures reached nearly the same level as RNA from RD-114 cells, from which the virus was produced.

Hybrid formation with various RNA preparations was completely abolished by pretreatment of the mixtures with RNase at 50 μg/ml. The cellular RNA preparations were routinely checked for DNA by Cs_2SO_4 density equilibrium centrifugation with negative results.

When the DNA probe was prepared in the presence of ^{3}H-dCTP, instead of ^{3}H-TTP, which was used in most cases, similar levels of hybridization were obtained, ruling out the possibility that the hybridization was based on specific artifacts due to the polymerization of TMP, e.g., hybridization with a Poly A stretch in cellular RNA (31). As further evidence of this assertion, neither Poly A nor Poly G at a concentration of 0.2 mg/ml hybridized with the DNA probe.

When the RD-114 viral 70*S* RNA (0.09 μg) was hybridized with the RD-114 DNA probe in the presence of excess of RD or Crandell cellular RNA (154 μg and 34 μg, respectively), no change of the hybridization levels was observed, indicating the absence of any interfering substances in the RNA preparations (data not shown).

Kinetics. When a fixed amount of the RD-114 DNA probe was hybridized with increasing amounts of various cat cellular RNAs, the reactions approximated second-order kinetics (23). To measure concentration of viral sequences in various RNAs, hybridization was carried out in the presence of excess amounts of RNA and the conversion of the DNA probe into hybrid form with time was assayed by S-1 nuclease. The data were plotted as the percentage of the hybridization (compared with the level of the hybridization obtained with a saturating amount of RD-114 viral 70*S* RNA) against the logarithm of the product of the initial molar concentration of RNA and the time of incubation (Cr·t). Such kinetic curves obtained with RNA from several cat cells are shown in Fig. 2. Also shown is a kinetic curve of RD-114 viral 70*S* RNA (Fig. 3). All curves fall nearly parallel to each other, indicating that the hybridization proceeded at a constant rate with these RNAs.

TABLE 3. *Cellular content of RD-114-specific RNA*

Origin of RNA	$(Cr \cdot t)_{1/2}$	Ratio[a] of initial rates	Number of copies per cell
RD-114 virus	4.4×10^{-3}	1.0	
RD-114 cells	3.0	14.7×10^{-4}	865
Liver tumor	22	2.0×10^{-4}	118
Crandell	32	1.37×10^{-4}	81
FL-74	35	1.26×10^{-4}	74
Embryo	43	1.02×10^{-4}	60
Liver (I)	78	0.564×10^{-4}	33
Liver (II)	660	0.067×10^{-4}	4

[a] Compared to viral RNA taken as 1.0.

As with DNA-DNA annealing, RNA-DNA hybridization in excess RNA can be characterized by a parameter designated $(Cr \cdot t)_{1/2}$ which is the value of Cr·t at half saturation (5, 6). At a given RNA input, the rate of hybridization was directly proportional to the concentration of homologous sequences in the RNA population, and thus the $(Cr \cdot t)_{1/2}$ of various RNAs was used to measure the concentration of the homologous RNA in comparison with that obtained with the RD-114 viral RNA. The values thus calculated are given in Table 3. The number of copies homologous to the RD-114 viral RNA per cell was calculated by assuming that a molecule of viral 70*S* RNA contains 1.7×10^{-11} μg (molecular weight $\sim 10^7$) and that the cellular RNA content is 10^{-5} μg/cell.

RD-114 cells contained about 0.15% of the total RNA as viral-specific RNA, corresponding to about 900 copies of the viral 70*S* RNA per cell. Cat liver tumor and Crandell cells contain the RD-114-specific RNA at about one-eighth and one-tenth of RD-114 cells, corresponding to about 120 and 80 copies per cell, respectively. The content in normal livers varied greatly, and one sample did not reach saturation at the highest RNA concentration used.

In the hybridization between DNA and large excess of homologous RNA, the complexity of the base sequences of the hybrids (number of the base pairs in the unique sequences per cell or virion) is directly proportional to the $(Cr \cdot t)_{1/2}$ (5, 6), and the molecular weight of the participating molecules can be estimated from the kinetic curves of the hybridization. Since known kinetic standards under the conditions used were not available, the complexity or the total molecular weight of the DNA probes cannot be estimated from our data, but the single-stranded DNA probes used in this study seem to cover a significant portion of the viral RNA from the following arguments. (i) Single-stranded DNA fractionated from the reaction products made in the presence of high concentration of actinomycin D shown similar kinetic curves with the 70*S* RNA as the DNA probe prepared from the 70*S* RNA hybrids, which were used in this study (Fig. 3). The single-stranded DNA made in the presence of actinomycin D is known to consist of a homogene-

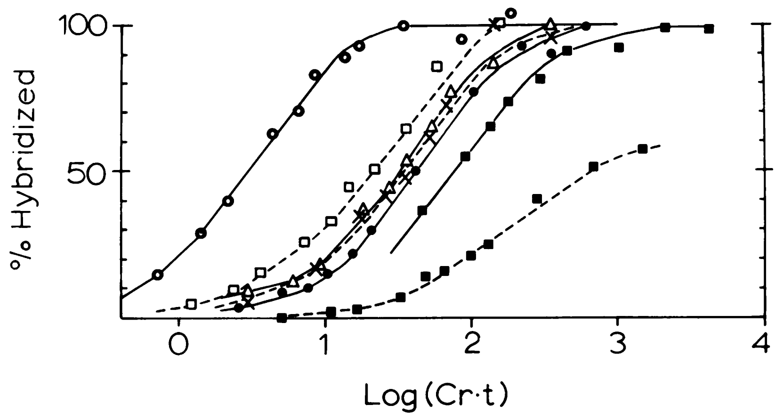

FIG. 2. *Kinetics of hybridization of cellular RNA with ³H-DNA product of RD-114 virus. ³H-DNA enzymatic product (620 counts/min) was mixed with various cellular RNA species in 50 µliters of annealing buffer at 67 C for various times. The extent of hybridization was measured after S-1 nuclease digestion and is expressed as the fraction of ³H-DNA hybridized with saturating amounts of RD-114 viral 70S RNA as determined in parallel incubations. The normalized hybridization value is plotted against log(Cr·t) calculated from total cellular RNA concentration and the time of incubation. Cellular RNA species used were: RD-114 (○), 0.36 mg/ml; cat hepatoma (□), 0.61 mg/ml; Crandell cell, CC (△); 1.49 mg/ml; F1-74 (FeLV producing cell line) (×), 1.44 mg/ml; cat embryo fibroblast (●), 0.43 mg/ml; cat liver-1 (■——■), 7.63 mg/ml; cat liver-2 (■-----■), 2.75 mg/ml.*

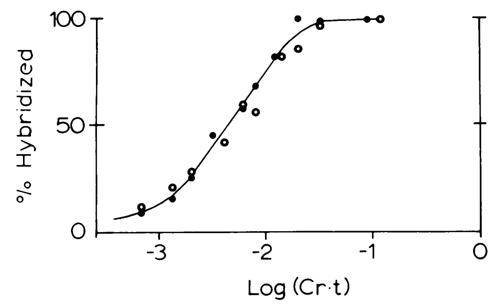

FIG. 3. *Kinetics of hybridization of RD-114 viral RNA with ³H-DNA enzymatic product of RD-114 virus. A fixed amount of RD-114 viral 70S RNA (0.338 µg/ml) was annealed with two kinds of ³H-DNA products: single-stranded DNA purified from the viral 70S-RNA-hybrids by sedimentation (○), (620 counts/min) and single-stranded DNA purified from the total reaction mixture with 100 µg actinomycin D per ml by hydroxyapatite fractionation (●), (530 counts/min). Hybridization was carried out in 50 µliters of the annealing buffer at 67 C for a given time, and expressed as in Fig. 2. Both sets of results are described by a single curve.*

ous population of the transcripts of the entire genome (12). (ii) Single-stranded DNA products in hybrid form seem to contain sequences homologous to the entire RNA genome (37). (iii) Large parts of the viral 70S RNA are known to be protected from the RNase hydrolysis when hybridized with excess of the single-stranded DNA product isolated from the homologous reaction mixture (10, 12). Similar experiments in our laboratory showed that a minimum of 58% of ³²P-labeled RD-114 70S RNA was protected from S-1 nuclease attack after hybridizing with 50-fold excess of single-stranded DNA product purified from the reaction mixture with 100 µg of actinomycin D per ml.

Size of the RD-114-specific RNA in cat cells. The RD-114-specific RNA in cat cells is present in a rather high amount, and can be easily detected by hybridization techniques. This enabled determination of the intracellular states of the RNA. Tsuchida et al. (36) have detected two discrete classes of viral specific RNA (35*S* and 20*S*) in MSV-transformed mouse and rat cells. When the RNA extracted by the hot phenol method was sedimented through a glycerol gradient and the hybridizability of each fraction was assayed with the RD-114 DNA probe, two distinct peaks of RD-114-specific RNA of about 35*S* and 18*S* were detected (Fig. 4). The distribution of these two classes of RNA was different with the cell types; Crandell cell had more 35*S* RNA than 18*S*, whereas cat embryo fibroblasts had 18*S* as the predominant population. This distribution is not due to aggregate formation because when the same RNA was sedimented through glycerol and DMSO-gradients, essentially the same patterns were obtained by both methods (Fig. 5). However, the virus producing RD-114 cells showed no such distinctive peaks of the virus-specific RNA, rather it was distributed throughout the gradient (Fig. 6), indicating a very heterogeneous population with respect to size. The fractions in brackets (I and II) were pooled, precipitated

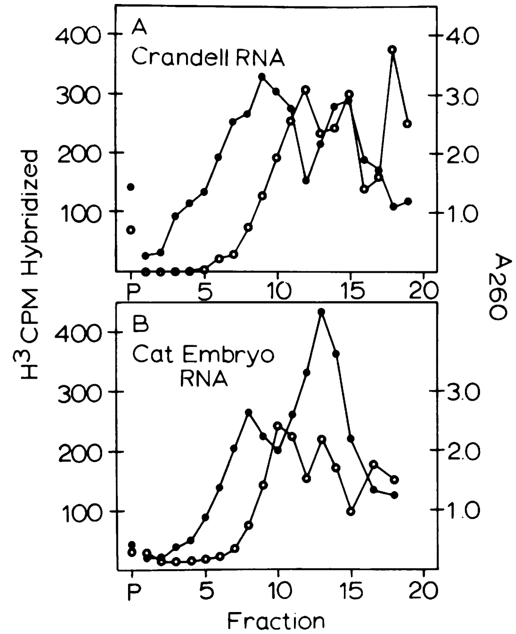

FIG. 4. *Sedimentation profiles of virus-specific RNA in cat cells. RNA was extracted from Crandell cells, CRFK, (A, 0.75 mg) and cat embryo fibroblasts (B, 0.47 mg) by the SDS-hot phenol method and centrifuged through 15 to 30% glycerol gradients at 24,000 rpm for 12 h (A) or 14 h (B) in the SW 41 rotor at 4 C. Absorbance at 260 m (O) was determined for each fraction and 75-µliter samples of each fraction were hybridized with ^{3}H-DNA product (1,100 counts/min) of RD-114 virus. Hybridized ^{3}H-DNA (●) was measured by S-1 nuclease digestion. Background counts obtained without added RNA (88 counts/min) were subtracted. Sedimentation from right to left. P denotes the pellets resuspended in 0.5 ml of TSE.*

with ethanol in the presence of yeast RNA, redissolved in 0.1 × SSC, and increasing amounts of each fraction were hybridized with the DNA probe; the extent of hybridization approached completion. Thus, the RD-114-specific RNA population seemed to be the same in each size fraction.

RNA from the FL-74 cell was fractionated by glycerol gradient centrifugation, and portions of each fraction were hybridized with the RD-114 and FeLV DNA probes. While RD-114-specific RNA was present in two discrete peaks around 35S and 18S, RNA specific for FeLV was distributed heterogeneously throughout the gradient (Fig. 7). The heterogeneity of the viral RNA in virus-producing cells was also reported with RSV-infected chicken cells (18). Whether this reflects the dynamic state of viral precursor RNA in virus-producing cells remained to be answered, but the viral RNA in immature virus particles harvested shortly after the release was reported to be quite heterogeneous in size and to become homogeneous during the maturation stage (8).

DISCUSSION

The above results clearly indicate that feline cells contain significant amounts of RNA which is complementary to the RNA of RD-114 virus.

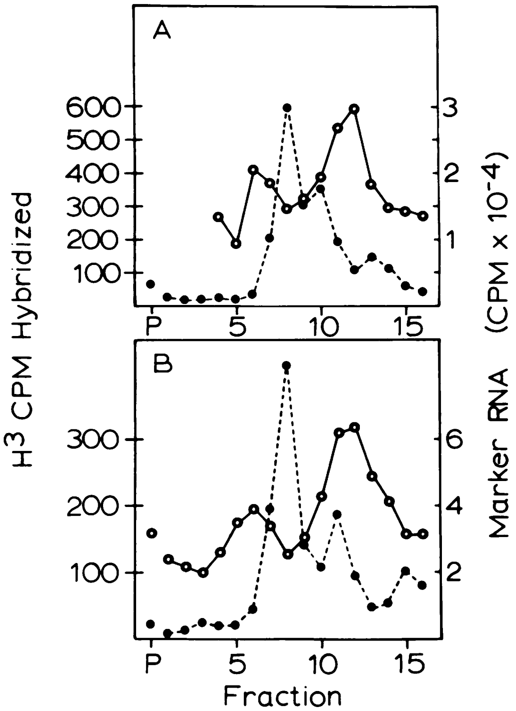

FIG. 5. *Sedimentation profiles of RD-114 viral-specific RNA from cat embryo cells. (A), DMSO gradient analysis. RNA (0.1 mg) purified from cat embryo cells was centrifuged through 4.6 ml of a linear gradient of 89% DMSO, 10% U-^{2}H-DMSO, 1% 1mM EDTA to 10% DMSO, 89% U-^{2}H-DMSO, containing 10% (wt/vol) sucrose and 1% 1mM EDTA at 40,000 rpm for 13 h at 25 C in a Spinco SW-65 rotor. The fractions, collected from the bottom, were precipitated with 2 volumes of ethanol in the presence of 50 µg of calf thymus DNA and 0.2 M NaCl. The precipitates were collected by centrifugation, rinsed with 70% ethanol, redissolved in 75 µliters of 0.1 × SSC, and hybridized with ^{3}H-DNA product (1,200 counts/min) of RD-114 viral enzyme (O). RD-114 cellular RNA, uniformly labeled with ^{3}H-uridine, was sedimented in a parallel run as a marker (●); (B), Glycerol gradient analysis. 0.2 mg of cat embryo RNA as also in (A) was centrifuged through a 15 to 30% (wt/vol) linear glycerol gradient at 26,000 rpm for 12 h in Spinco SW-41 rotor, and 75 µliters of each fraction was hybridized with ^{3}H-DNA product (1,200 counts/min) of RD-114 viral polymerase as described in Fig. 4.*

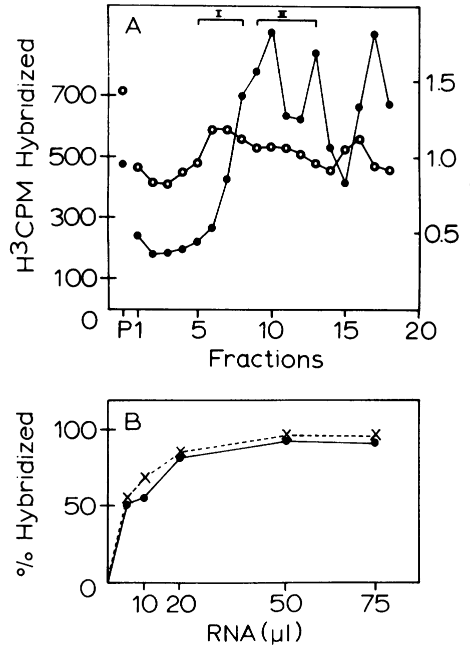

FIG. 6. *Sedimentation profile of RD-114 viral-specific RNA in RD-114 cells. (A), RNA (0.39 mg) purified from RD-114 cells was centrifuged through a 15 to 30% (wt/vol) glycerol gradient at 25,000 rpm for 13 h in a Spinco SW 41 rotor, and 75 µliters of each fraction was hybridized with 900 counts/min of RD-114 ^{3}H-DNA product as described in Fig. 4. Background of 82 counts/min with no RNA present was subtracted. ^{3}H-counts per minute hybridized (O); A_{260} (●). (B), Fractions in brackets, I and II, of the above gradient were pooled, precipitated with 2 volumes of ethanol in the presence of 50 µg of yeast RNA and 0.2 M NaCl, washed with 70% ethanol, and redissolved in 0.4 ml of 0.1 × SSC. Increased amounts of each pool were hybridized with the ^{3}H-DNA product of RD-114 viral polymerase. The amount of ^{3}H-DNA hybridized was normalized for the value obtained with 0.09 µg of RD-114 viral 70S RNA (750 counts/min) after subtraction of the background. Symbols: pool I, ●; pool II, ×.*

The RNA was detected by hybridization as sequences complementary to the viral DNA product. The technique is very sensitive and specific, and the virus-specific RNA is only detected in cells infected with RD-114 virus and cat cells. The RNA sequences homologous to the RD-114 virus RNA seem to be quite ubiquitous in cat cells, ranging from normal organs to established lymphocytic cell lines.

Parks et al. (25) briefly described the presence of RLV-specific RNA sequences in normal NIH Swiss 3T3 cells detected by a similar technique. The cells were also shown to contain very low levels of the viral gs antigen only detectable by a radioimmunoassay. Avian virus-specific RNA sequences were also detected and quantitated in normal chicken embryo cells which contained the viral gs antigen or the virus-related helper factor, but not in cells which lacked these markers (16). Apparently, not all of the viral RNA sequences are present in these cells, judging from the lower level of the hybridization attained. In the RD-114 feline cell system, however, the virus-specific RNA sequences were detected in all cat cells so far examined, and the level of the hybridization attained was the same as that obtained with the viral 70S RNA. These cat cells showed no sign of the presence of the virus-specific proteins when assayed by the complement-fixation test.

The difference in size distribution between viral RNA of actively producing and normal cells appears to offer some possibilities for understanding the lack of particle production by normal cat cells. It appears quite striking that the cells actively producing virus (either FeLV or RD-114) show marked heterogeneity, whereas the normal cat cell RD-114 RNA was distributed mainly in 35S and 18S size classes. The FL-74 cell provided a unique opportunity to demonstrate the reality of this observation since it contains both FeLV RNA with a heterogeneous distribution and the RD-114 RNA pattern characteristic of normal cat cells.

The finding that the FL-74 cell contains RD-114 RNA has pointed up one potential artifact in inter-viral hybridization studies. Thus, while RD-114 and feline Type C viruses

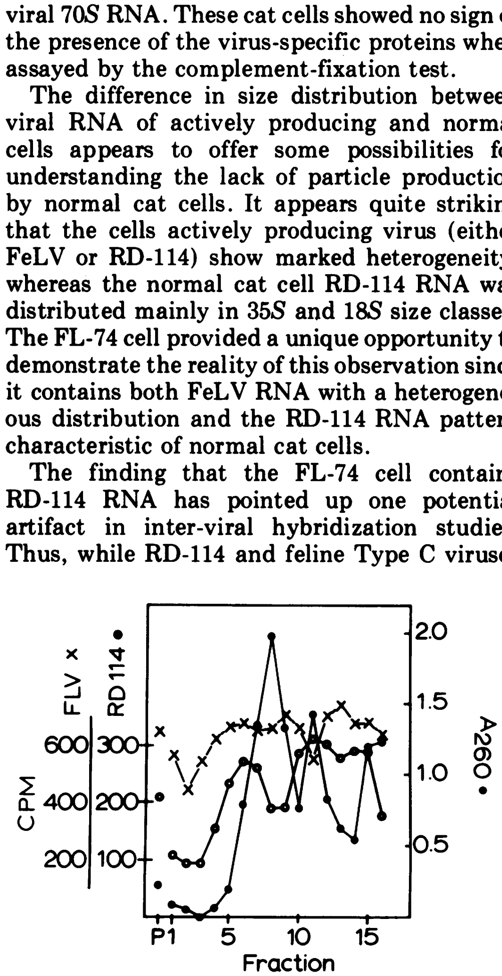

FIG. 7. *Sedimentation profiles of RD-114 and FeLV-viral-specific RNA in the FeLV producing FL-74 cells. A 0.44-mg amount of purified FL-74 cellular RNA was centrifuged through a 15 to 30% (wt/vol) glycerol gradient at 26,000 rpm for 12 h in a Spinco SW 41 rotor, and of 75 and 50-µliter samples of each fraction hybridized with ^{3}H-DNA products of RD-114; 1,100 counts/min (O) and FeLV, 780 counts/min (×), respectively. Backgrounds (113 counts/min for RD-114 ^{3}H-DNA, 42 counts/min for FeLV ^{3}H-DNA) were subtracted. Absorbance at 260 nm, (●).*

are readily distinguished by molecular hybridization, a significant degree of cross-reaction was seen when RD-114 DNA probes were tested against FeLV of cat cell origin. When the FeLV viral RNA was obtained from virus grown on human cells, essentially no cross-hybridization was observed. This indicates the strong possibility that RD-114 RNA may be included in FeLV preparations. This type of artifact may influence results when viruses within a species are compared after growth in host cells of that same species.

The finding of viral RNA in normal cells of three species indicates that the hybridization technique (viral DNA-cell RNA) is useful in studies of the origin of specific type C viruses.

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